ePHOGSY experiments on a paramagnetic protein: location of the catalytic water molecule in the heme crevice of the oxidized form of horse heart cytochrome *c*

Ivano Bertini[a,*], Claudio Dalvit[b], J. Gaspard Huber[a], Claudio Luchinat[c], Mario Piccioli[a]

[a]*Department of Chemistry, University of Florence, Via Gino Capponi 7, I-50121 Florence, Italy*
[b]*Novartis Pharma AG, CH-4002 Basel, Switzerland*
[c]*Department of Soil Science and Plant Nutrition, University of Florence, Florence, Italy*

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Abstract The hydration properties of the oxidized form of horse heart cytochrome *c* have been studied by ^{1}H NMR spectroscopy. Application of ePHOGSY (enhanced protein hydration observed through gradient spectroscopy) experiments over a paramagnetic molecule provided firm spectroscopic evidence of the presence of a water molecule in the heme crevice. A few intermolecular NOEs have been used to locate the water molecule at about 0.65 nm away from the iron atom and to compare the position observed in solution with that observed in the crystal structure and in solution for the reduced state. The resulting picture is that there is a detectable movement of the water molecule upon oxidation.

Key words: Cytochrome *c*; Protein hydration; Paramagnetic NMR

1. Introduction

Structure and hydration properties of cytochromes *c* (cytc) are actively debated. For both aspects, oxidation state dependent changes are supposedly relevant to the protein function. They have been addressed by several methodologies with, in some case, discrepant results [1–7] (Banci et al., personal communication).

In particular, the localization and even the presence of a water molecule in the distal heme crevice are matter of discussion. Crystallographic studies performed on both oxidation states for tuna [8,9] and *S. cerevisiae* cytc [5,10] pointed out the possible role of water molecules in the heme crevice. Berghuis and Brayer described a 0.17 nm decrease in the Fe-oxygen distance of the water closer to the heme upon oxidation [5]. This was accomplished by a different orientation of the water dipole with respect to the heme and by alterations in the surrounding hydrogen-bonded networks. These findings lead to propose a specific role of a water molecule in stabilizing both oxidation states via a different localization of the water molecule in the heme crevice. Due to its proposed role this water molecule is commonly referred to as 'catalytic' water.

*Corresponding author. Tel.: +39 (55) 2757549;
Fax: +39 (55) 2757555.
E-mail: bertini@risc1.lrm.fi.cnr.it

Abbreviations: ePHOGSY, enhanced protein hydration observed through gradient spectroscopy; cytc, cytochrome *c*; hh, horse heart; REM, restrained energy minimization

Protein hydration studies are a challenging aspect of contemporary bimolecular NMR [11–13]. Following pioneering work of Otting and Wütrich [14,15], several possible approaches have been proposed, based on isotope labeling [16], pulsed field gradients [17,18] and any combination of these methods [19]. We approached the problem using one- and two-dimensional NMR experiments based on the so called ePHOGSY scheme, originally developed by Dalvit [20,21] and we took into account the paramagnetic properties of the oxidized horse heart (hh) cytochrome c. We report here firm spectroscopic evidences to identify, in solution, the occurrence of the catalytic water molecule in the distal cavity of the protein. Several protein-water NOEs locate the water molecule close to propionate 7, about 0.65 nm away from the iron atom.

2. Materials and methods

Horse heart cytochrome *c* (Type VI) was obtained from Sigma Chemical Co. and used without further purification. The ^{1}H NMR samples were prepared by dissolving the lyophilized protein in 50 mM phosphate buffer at pH 5.7 to give 10–15 mM solutions.

^{1}H NMR spectra were recorded, at 293 K, on a Bruker AMX 600 spectrometer, equipped with a BGU unit. A 5 mm probe with self shielded z-gradients was used.

In all experiments, a mixing time of 120 ms was used for both water-protein and protein-protein transfer [20]. The recycle delay was 1.5 s. To suppress solvent signal, a Watergate filter was used before acquisition [17]. The binomial pulse sequence 3-9-19 was used [22]. The TPPI method was used to obtain quadrature detection in the F1 dimension [23]. Gaussian shaped, selective 180° pulses of 15 or 51 ms were applied on resonance with the carrier frequency at the H_2O position. The 180° selective pulse and the choice of power level for gradient pulses constitute critical parameters to set up experiments. The experiment with 51 ms 180° pulse was used to check whether proton signals close to the water (mostly H_α protons) have been excited. In all two-dimensional experiments a spectral window of 8400 Hz was used. In the NOESY experiment, a total of 48 transients were collected for a 2048×870 data points matrix. In the case of ePHOGSY-NOESY experiments, a total of 640 transients were collected for a 1024×286 data points matrix. Prior to zero filling (2048×1024 for the NOESY, 1024×512 for the ePHOGSY-NOESY) and Fourier transformation, a squared cosine bell window function was applied in both dimensions.

Restrained energy minimization (REM) calculations were performed using the SANDER [24] module of the AMBER4 program package [25].

3. Results and discussion

ePHOGSY is a pulse scheme that acts as a filter for water-protein dipolar interactions [20,21]. Because the intensity of ePHOGSY-NOESY cross peaks depends on both H_2O-pro-

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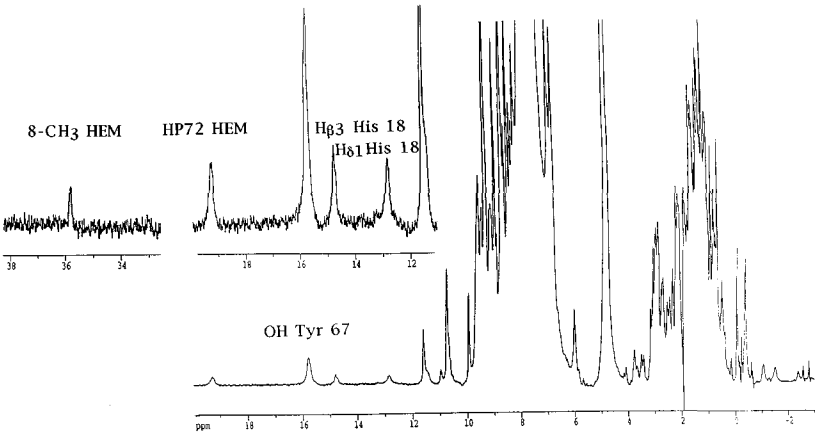

Fig. 1. 600 mHz, one-dimensional ePHOGSY experiment recorded at 120 ms mixing time. A total number of 38912 scans was collected. To avoid any possible contribution arising from exchange peaks the signal corresponding to the OH^- resonance of Tyr 67 has not been taken into consideration.

tein and protein-protein dipolar interactions, for each of the assigned cross peaks the intensity ratio between ePHOGSY-NOESY and NOESY – provided that the second mixing of ePHOGSY-NOESY is the same of the reference NOESY – is directly dependent on the water-protein NOE.

About 700 cross peaks have been assigned in the ePHOGSY-NOESY spectra. They correspond to NOESY cross peaks between protein-proton pairs. The intensities of the cross peaks on each row scale down according to the NOE (or chemical exchange) between water and the protein proton identified on the intersection of the diagonal with each row. The $I_{ePHOGSY-NOESY}/I_{NOESY}$ ratios gave a set of 241 water-protein interactions, of which 204 are NOEs to non-exchangeable protein protons.

In order to obtain additional information arising from paramagnetically shifted signals, one-dimensional version of the ePHOGSY experiment has been recorded over a large spectral window (48 kHz, Fig. 1). Analogously to what extensively discussed when dealing with 1D vs. 2D NOE in paramagnetic systems [26], 1D ePHOGSY ensures a large S/N ratio within a reasonable experimental time and permits the observation of ePHOGSY peaks on the hyperfine shifted signals that could not be detected in conventional 2D ePHOGSY experiments. Six additional connectivities were observed in the region of hyperfine shifted signals, two of which with heme proton resonances, $H_{\alpha 2}$ of propionate 7, at ca. 19 ppm, and the 8-CH_3 methyl resonance at ca. 36 ppm. The 1D NOEs were scaled with 2D NOEs by using isolated peaks with good signal to noise ratio visible on both spectra.

Some of the water-protein NOEs could arise from off-resonance effects on protons close in chemical shift to the water resonance (typically H_α protons). Therefore, in order to calibrate experimental water-protein NOEs, we assumed that the most intense constraints in the set of 209 constraints which correspond to non-exchangeable protons and whose chemical shifts are not in the 4.5–5.1 ppm range, is at 0.25 nm from a water molecule. Then, the weakest constraints originating from the 2D spectrum give an estimated distance of 0.6 nm. We increased these values by a factor of 20% in order to take into account the possible experimental errors.

The observation of NOEs originating from a hypothetical water molecule located in the heme crevice may be complicated by the presence of other water molecules, for example close to Trp 59, and by the already mentioned off-resonance effects on protons close in chemical shifts with the water resonance. Keeping this in mind and considering also the available X-ray structure that shows a water molecule in the crevice [7], the constraints shown in Table 1 have been selected.

The experimental distance constraints were used to calcu-

Table 1
Non-exchangeable protons within a 0.55 nm sphere from the catalytic water molecule in the REM molecule and experimental upper limit distances used for calculations

Protons	Upper limit distances (nm)	REM derived distances (nm)	X-ray derived distances (nm)[a]
HP71 HEM	0.432	0.310	0.401
HP72 HEM	0.504	0.371	0.490
8-CH_3 HEM	0.912	0.500	0.683
H_h Trp 59	0.276	0.268	0.545
H_{z1} Trp 59	0.576	0.225	0.483
H_{b2} Tyr 67	0.528	0.497	0.720
g-CH_3 Ile 75	0.408	0.418	0.452
H_{b2} Asn 52	0.440[b]	0.424	0.268
H_b Ile 75	0.384[b]	0.362	0.291
H_b Thr 78	0.396[b]	0.531	0.307
g-CH_3 Thr 78	0.400[b]	0.408	0.301

[a]All protein proton-water proton distances, r, are expressed as 'reduced' distances due to the presence of two protons on the water molecule: $r = (1/r_A^6 + 1/r_B^6)^{-1/6}$ where r_A and r_B are the distances from the protein p223roton and the two protons of the catalytic water.
[b]Not used in the minimization (see text).

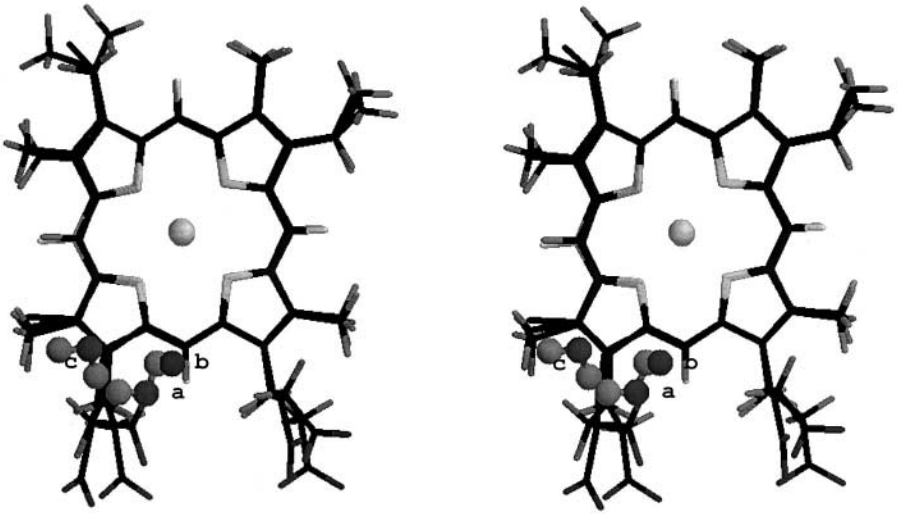

Fig. 2. Stereo view of catalytic water molecules with respect to the heme in: the solution REM structure of oxidized form (a, this work); the X-ray structure of the oxidized form [4] (b); and solution structure of reduced form of horse heart cytochrome *c* [2] (c). The ring atoms of the heme and the iron atom of each structure were used for the superposition.

late the structure in solution of hh cytc plus a water molecule arbitrarily placed outside the distal heme cavity through restrained energy minimization (REM). Only two constraints, originating from $H_{\beta 2}$ Tyr 67 and H_{γ} Ile 75, provide small violations of upper limit distances (less than 8×10^{-3} nm) after the REM process.

The experimental restraints drive the water molecule toward a minimum energy position rather different from what observed through X-ray analysis (Table 1). Indeed, the catalytic water still occupies the distal cavity of oxidized hh cytc but, at variance with all X-ray studies reported so far [5–10], it is not symmetric with respect to the two propionate groups but definitely closer to propionate 7. The water molecule is about 0.12 nm, with respect to the oxidized X-ray structure [4], and about 0.25 nm with respect to the reduced solution structure [2] (Fig. 2). The position of water is also consistent with four additional NOEs which we have not used for the REM process (Table 1).

The REM process has been replicated starting from two water molecules inside the cavity in different positions. It appears that only one water molecule takes a position which satisfied the restraints. Thus, our experimental data are fully consistent with only one water molecule.

In our minimized structure, the water is stabilized by a H-bond with Tyr 67 OH, and by the presence of the hydrophilic residue Thr 78, although there is no direct H-bond with Thr 78 OH. The possibility of a H-bond with Asn 52 cannot be ruled out. The orientation of the amidic side chain group of Asn 52 is poorly defined in solution. However, ePHOGSY experiments reveal that at least one H_{δ} Asn 52 is near or in exchange with a water molecule.

4. Conclusion

We have provided the first NMR evidence of the 'catalytic' water molecule in the oxidized form of hh cytc in solution.

The solvent molecule is trapped in the heme crevice with residence times longer than the rotational correlation time of the protein and shorter with respect to the NMR chemical shift time scale. It is therefore expected that its lifetime inside the protein is $10^{-9}\ \mathrm{s} < t < 10^{-3}$ s.

The water molecule is localized in the heme crevice corresponding to the distal position, and located in close proximity to the heme propionate 7. The difference between the position observed in solution with that observed in the X-ray structure exceeds the experimental errors of both techniques. Furthermore, differences beyond uncertainty are also detected for the catalytic water between oxidized and reduced forms [3]. The proposed stabilization of the oxidized form of cytc due to the reorientation of the water dipole upon oxidation should be reconsidered in the light of the present data. A final remark is that the orientation of the water dipole is not directly accessible experimentally, because X-ray data only provide the location of the oxygen atom and NMR the average location of the hydrogens. The subtle differences between X-ray and solution structures at the present resolution do not permit a safe merging of the two sets of data at this stage.

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